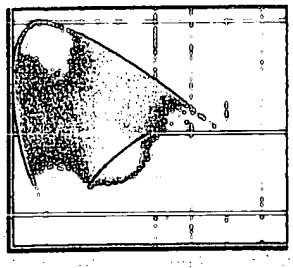
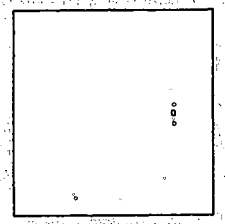
Chloroplast engineering with operons

Most agronomic traits result from the action of several genes, so efficient strategies to express multiple genes in transgenic plants are sorely needed, and not at all trivial undertakings. In this issue, Daniell and colleagues show a way to stably integrate into the chloroplast genome the entire *Bt cry*2Aa2 operon, which codes for production of an insecticidal protein, as well as accessory proteins to help with folding. The insecticidal protein accumulated to extremely high levels in leaves (~35% of total soluble protein) in a crystalline form, and insects recalcitrant to other control methods were totally wiped out after munching on leaves from these plants. This successful expression of multiple genes through a single transformation event may make multigene expression of foreign pathways or pharmaceutical proteins less of a chore (see p. 71). *JJ*



On page 62, Jenison *et al.* describe a biosensor for detecting nucleic acid targets present at only attomolar concentrations. The detector comprises a set of oligonucleotide probes bound to a silicon-based surface that can hybridize to specific nucleic acid targets. Bound nucleic acids are then detected by complementary probes labeled with biotin, which catalyzes an enzymatic reaction that deposits a thin film on the surface of the silicon. The color change that results on the biosensor's surface is detectable by the naked eye, suggesting potential applications in the clinic and field. *MS*



A screen for ribozyme inhibitors

Catalytic RNA is increasingly viewed as a promising drug target, particularly in that some catalyze activities specific to microorganisms and viruses. On page 56, Jenne *et al.* describe a fluorescence-based assay to find inhibitors of such catalytic RNA. They used a high-throughput FRET-based assay that reports the activity of a hammerhead ribozyme. The ribozyme cleaves a specially designed RNA molecule holding together a fluorophore and a quencher dye, resulting in a fluorescent signal that is reduced by inhibitors of the ribozyme. They used the assay to screen a library of 96 known antibiotics, and extracts of nearly 2,000 different actinomycete strains, and detected a number of inhibitory compounds with potential antibiotic activities, one of which they validated as effective *in vivo*. They estimate that even without automation such an assay could analyze on the order of 500 different ribozyme reactions per day to search for new antibiotics. *ND*

Overexpression of the *Bt cry*2Aa2 operon in chloroplasts leads to formation of insecticidal crystals

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In nuclear transgenic plants, expression of multiple genes requires introduction of individual genes and time-consuming subsequent backcrosses to reconstitute multi-subunit proteins or pathways, a problem that is compounded by variable expression levels. In order to accomplish expression of multiple genes in a single transformation event, we have introduced several genes into the chromoplast genome. We confirmed stable integration of the *cry*2Aa2 operon by PCR and Southern blot analyses in T_0 and T_1 transgenic plants. Foreign protein accumulated at 45.3% of the total soluble protein in mature leaves and remained stable even in old bleached leaves (46.1%), thereby increasing the efficacy and safety of transgenic plants throughout the growing season. This represents the highest level of foreign gene expression reported in transgenic plants to date. Insects that are normally difficult to control (10-day old cotton bollworm, beet armyworm) were killed 100% after consuming transgenic leaves. Electron micrographs showed the presence of the insecticidal protein folded into cuboidal crystals. Formation of crystals of foreign proteins (due to hyperexpression and folding by the putative chaperonin, ORF 2) provides a simple method of purification by centrifugation and enhances stability by protection from cellular proteases. Demonstration of expression of an operon in transgenic plants paves the way to engineering new pathways in plants in a single transformation event.

Keywords: polycistrons, plastid transformation, GM crops, *Bt* resistance

In plant and animal cells, the monocistronic translation of nuclear messenger RNAs (mRNAs) poses problems in engineering multiple genes in plants[1]. To express the polyhydroxybutyrate polymer or Guy's 13 antibody, for example, single genes were first introduced into individual transgenic plants, then these plants were backcrossed to reconstitute the entire pathway or the complete protein[2,3]. Similarly, in a seven year long effort, Ye *et al.*[4] recently introduced a set of three genes for a short biosynthetic pathway that resulted in β-carotene expression in rice. In contrast, most chloroplast genes of higher plants are co-transcribed[1]. Multiple steps of chloroplast mRNA processing are involved in the formation of mature mRNAs. Expression of polycistrons via the chloroplast genome provides a unique opportunity to express entire pathways in a single transformation event. Additionally, chloroplast genetic engineering is an environmentally friendly approach resulting in containment of foreign genes and hyperexpression[5,6].

In this study, the *Bacillus thuringiensis* (*Bt*) *cry*2Aa2 operon is used as a model system to demonstrate operon expression and crystal formation via the chloroplast genome. The *cry*2Aa2 is the distal gene of a three-gene operon. The open reading frame (ORF) immediately upstream of *cry*2Aa2 codes for a putative chaperonin that facilitates the folding of Cry2Aa2 (and other proteins) to form proteolytically stable cuboidal crystals[7–9]. Because Cry protein levels decrease in plant tissues late in the growing season or under physiological stress[10], a more stable protein expressed at high levels in the chloroplast throughout the growing season should increase toxicity of *Bt* transgenic plants to target insects and help eliminate the development of *Bt* resistance. Therefore, the *cry*2Aa2 bacterial operon is expressed in tobacco chloroplasts to test the resultant transgenic plants for increased expression and improved persistence of the accumulated insecticidal protein(s).

Results

Chloroplast vector. The 4.0 kb *cry*2Aa2 operon was inserted into the universal chloroplast expression vector pLD CtV2 (5.8 kb) to form the final *Escherichia coli* and tobacco shuttle vector pLD-BD *Cry*2Aa2 operon (9.8 kb) (Fig. 1A). This vector should be able to transform chloroplast genomes of several plant species because the flanking sequences are highly conserved among higher plants[11,12]. This vector contains the 16S ribosomal RNA (rRNA) promoter (*Prrn*) driving the *aadA* gene (aminoglycoside 3′- adenylyltransferase) for spectinomycin selection and the three genes of the *cry*2Aa2 operon. The terminator is the *psbA* 3′ region from the tobacco chloroplast genome from a gene coding for the photosystem II reaction center component. The 16S rRNA promoter is one of the strong chloroplast promoters recognized by both nuclear and plastid-encoded RNA polymerases in tobacco, and the *psbA* 3′ region stabilizes the transcript of foreign genes. This construct integrates both genes into the spacer region between the chloroplast transfer RNA genes coding for isoleucine and alanine within the inverted repeat (IR) region of the chloroplast genome by homologous recombination. The integration into these transcribed spacer regions allows the gene to be inserted without interfering with gene coding regions. Also, each gene will contain two gene copies as a result of

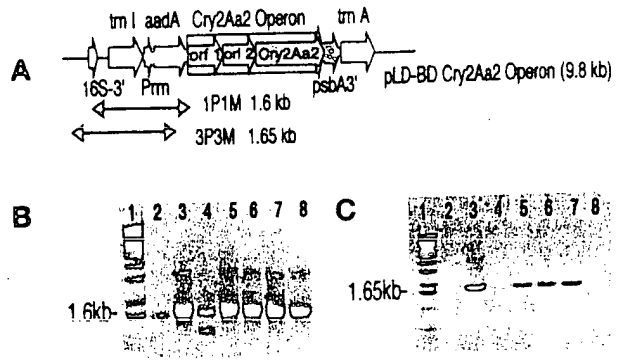

Figure 1. Chloroplast expression vector and PCR analysis. (A) pLD-BD *Cry2Aa2* operon (9.8 kb) with PCR primer binding sites and expected fragment sizes. PCR analysis of untransformed and putative chloroplast transformants using two primer sets: (B) 1P1M and (C) 3P3M. Lane 1, 1 kb ladder; lane 2, untransformed; lanes 3–7, pLD-BD Cry2Aa2 operon putative transformants; lane 8, pLD-BD Cry2Aa2 operon plasmid DNA.

integration into the two IR regions, resulting in a higher copy number (7,000–8,000 copies/cell) and higher levels of expression.

Chloroplast integration of foreign genes. Chloroplast transgenic plants were obtained as described[13,14]. Foreign gene integration into the chloroplast genome was determined by PCR screening of chloroplast transformants (Fig. 1 A–C). Primers were designed to eliminate spectinomycin mutants and nuclear integration. The first primer set, 1P1M, targets one primer (1P) to the 3′ end of the 16S rRNA flanking sequence and another primer (1M) to *aadA* (Fig. 1A). This is to distinguish between spectinomycin mutants and true spectinomycin transformants. A 1.6 kb fragment is seen in true transformants (Fig. 1B, lanes 3, 5–7). Lane 4 shows a spectinomycin mutant with no PCR product. Untransformed tobacco DNA (lane 2), as expected, shows no product, whereas pLD-BD *Cry2*Aa2 operon plasmid DNA in lane 8 produced the 1.6 kb fragment. The second primer set, 3P3M, targets one primer (3P) to the native chloroplast genome adjacent to the point of integration, and another primer (3M) on the *aadA* gene (Fig. 1A). This primer set generated a 1.65 kb PCR product in chloroplast transformants (Fig. 1C, lanes 3, 5–7). Untransformed tobacco DNA (lane 2) showed no PCR product, and pLD-BD *Cry2*Aa2 operon plasmid DNA in lane 8 also showed no PCR product because 3P binds to native chloroplast DNA. Lane 4 was negative for chloroplast integration, again proving this transformant to be a spectinomycin mutant.

Southern blot analysis was done to further demonstrate site-specific chloroplast integration of the 4.0 kb *cry2*Aa2 operon and to determine heteroplasmy or homoplasmy (Fig. 2). *Bgl*II digested DNA from transformed plants produce 8.42 and 1.4 kb fragments when probed with the 0.81 kb probe that hybridizes to the *trn*I and *trn*A flanking sequences. Transgenic plant DNA (T_0 and T_1) produced the 8.42 and 1.4 kb fragments (lanes 3–9). A 4.47 kb fragment is seen in untransformed plant DNA (lane 2). T_0 plant DNA also shows this native untransformed 4.47 kb fragment (lanes 3–7), thereby showing heteroplasmy in the T_0 generation. This 4.47 kb native band is absent from the T_1 generation (lanes 8, 9), thus indicating homoplasmy. If only a fraction of the genomes were transformed, the gene copy number should be <8,000 per cell. Confirmation of homoplasmy in T_1 transgenic lines indicates that the *cry2*Aa2 operon gene copy number could be as many as 7,000–8,000 per cell.

CRY2Aa2 protein expression and quantification. Expression profile of the operon-derived (OD) Cry2Aa2 and single gene-derived (SG) Cry2Aa2 (ref. 15) is shown on a Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (Fig. 3). The primary goal of this experiment is to investigate the location of the operon-derived Cry2Aa2 protein (the

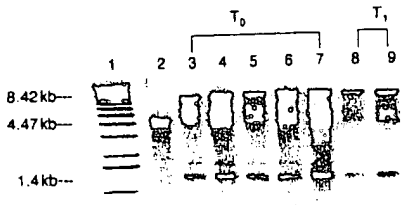

Figure 2. Southern blot analysis of T_0 and T_1 generations. Lane 1, 1 kb ladder; lane 2, untransformed; lanes 3–7, T_0 transgenic lines; lanes 8 and 9, T_1 transgenic lines.

pellet or supernatant) and correlate with cuboidal crystals observed in electron micrographs (see Fig. 6). Lane 2 contains partially purified 65 kDa Cry2Aa2 from *E. coli*. Because crystalline Cry2Aa2 inclusion bodies are solubilized at high alkaline pH (ref. 16), the 50 mM sodium hydroxide-solubilized pellet was analyzed from each plant sample after centrifugation for 20 min at 13,000 *g* (lanes 3, 5, 7). Results show that OD Cry2Aa2 expression forms crystalline inclusion bodies because the protein is found mostly in the pellet after centrifugation (lanes 5, 6). In contrast, expression of SG Cry2Aa2 is observed in both the pellet and the supernatant (lanes 3, 4). No Cry2Aa2 expression was seen in untransformed tobacco in either the supernatant or the pellet (lanes 7, 8).

Cry 2Aa2 polypeptides (lanes 3, 5) were scanned using Storm 840 Gel Scanner and Image Quant Software (Molecular Dynamics, Sunnyvale, CA). The OD expression results only in a 2.5-fold greater accumulation of Cry2Aa2 than that of SG-derived Cry2Aa2 in the pellet fraction; this does not correlate with more than 100-fold difference observed in enzyme-linked immunosorbent assay (ELISA; Fig. 4). The reason for this discrepancy is the extreme difference in solubilization between SG Cry2Aa2-derived amorphous inclusion bodies and the OD Cry2Aa2-derived cuboidal crystals, as reported previously[17,18]. Despite the large difference in protein accumulation (as shown by ELISA and electron micrographs, Figs 4, 6), the concentration of solubilized protein loaded in the pellet fraction was similar in SG Cry2Aa2 and OD Cry2Aa2 (Fig. 3, lanes 3, 5). Attempts to completely solubilize crystalline inclusion bodies for SDS–PAGE analysis were not successful because higher pH interfered with gel electrophoresis and repeated dilution decreased protein concentration below detectable levels in Coomassie-stained gels.

However, for quantification using ELISA it was possible to completely solubilize crystalline inclusion bodies under optimal conditions and dilute the protein to fit within the linear range of the Cry2aA2 standard. Therefore, protein expression levels of SG

Figure 3. 10% SDS–PAGE gel stained with R-250 Coomassie blue. Loaded protein concentrations are provided in parentheses. Lane 1, prestained protein standard; lane 2, partially purified Cry2Aa2 protein from *E. coli* (5 μg); lane 3, single gene-derived Cry2Aa2 pellet extract solubilized in 50 mM NaOH (22.4 μg); lane 4, single gene-derived Cry2Aa2 supernatant (66.5 μg); lane 5, operon-derived Cry2Aa2 pellet extract solubilized in 50 mM NaOH (22.9 μg); lane 6, operon-derived Cry2Aa2 supernatant (58.6 μg); lane 7, untransformed tobacco pellet extract solubilized in 50 mM NaOH (29.8 μg); lane 8, untransformed tobacco supernatant (30.4 μg). Colored compounds observed in the supernatant of transgenic plants interfered with the DC Bio-Rad protein assays.

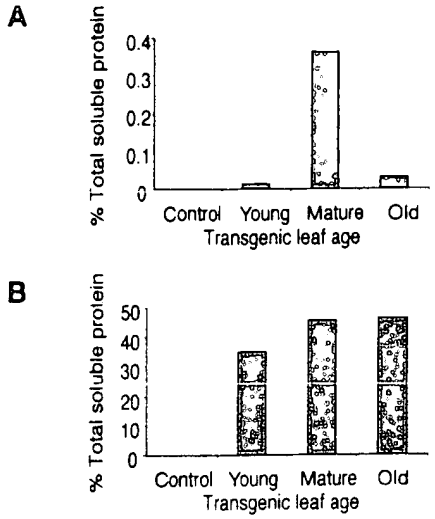

Figure 4. Protein quantification by ELISA in young, mature, and old transgenic leaves. (A) Single gene-derived Cry2Aa2 expression shown as a percentage of total soluble protein. (B) Operon-derived Cry2Aa2 expression shown as a percentage of total soluble protein.

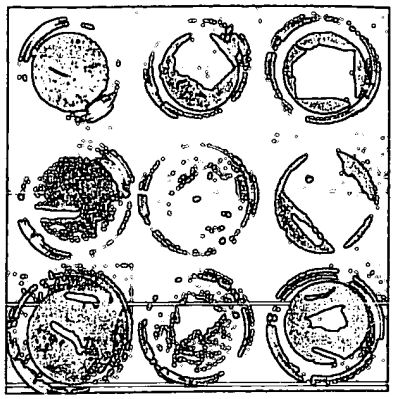

Figure 5. Insect bioassays. (A, D, G) Untransformed tobacco leaves; (B, E, H) single gene-derived Cry2Aa2 transformed leaves; (C, F, I) operon-derived Cry2Aa2 transformed leaves. (A–C) Bioassays with *Heliothis virescens*; (D–F) bioassays with *Helicoverpa zea*; (G–I) bioassays with *Spodoptera exigua*. All leaf samples for each replicate were from the same leaf. Two samples were evaluated per treatment, and observed daily for mortality and leaf damage for five days. Treatments were replicated three times. Insects were tested at 5 or 10 days old (see text for details).

Cry2Aa2 and OD Cry2Aa2 were quantified using ELISA (Fig. 4). Additionally, Cry protein accumulation in young, mature, and old transgenic leaves derived from a single gene or operon was compared to investigate their stability over time. Young, mature, and old leaves expressed SG Cry2Aa2 at 0.014%, 0.36%, and 0.03% respectively (Fig. 4A). Cry2Aa2 levels peaked in the mature leaf (0.36%) and drastically declined to 0.03% as the plant senesced. However, young, mature, and old leaves containing OD Cry2Aa2 accumulated at 34.1%, 45.3%, and 46.1%, respectively (Fig. 4B). As these transgenic plants aged, OD Cry2Aa2 concentrations remained stable and did not decline like the SG Cry2Aa2. The presence of the operon-expressed putative chaperonin should enable the toxin to be folded into stable crystalline structures that are protected from degradation. Based on quantitative expression, the *cry*2Aa2 operon-derived expression levels are comparable to those of the RuBisCo, the most abundant protein on earth that composes up to 65% of leaf soluble protein[19].

Insect bioassays: Five-day-old tobacco budworm (*Heliothis virescens*), 10-day-old cotton bollworm (*Helicoverpa zea*), and beet armyworm (*Spodoptera exigua*) insects consumed the entire leaf after 24 h on the untransformed control (Fig. 5A, D, G). When feeding on SG Cry2Aa2 leaves, *H. virescens* insects died after five days (Fig. 5B) whereas insects died after three days on OD Cry2Aa2 leaves (Fig. 5C). For SG Cry2Aa2, *H. zea* insects had consumed considerable leaf material after 24 h, stopped feeding after three days, and died after five days (Fig. 5E). For OD Cry2Aa2, *H. zea* insects had consumed very little material after 24 h, stopped feeding, and died after five days (Fig. 5F). When feeding on SG Cry2Aa2 (Fig. 5H) or OD Cry2Aa2 (Fig. 5I), *S. exigua* were lethargic after 24 h and died after 48 h.

Milkweed leaves dusted with OD Cry Aa2 transgenic pollen were not toxic to monarch butterfly insects (data not shown), confirming earlier observations that foreign proteins are not present in tobacco pollen[5].

Electron microscopic (EM) analysis. Untransformed and transgenic leaf sections were immunogold-labeled with a Cry2A polyclonal antibody (Fig. 6). Figure 6A–C shows developmental OD Cry2Aa2 in chloroplasts in young, mature, and old leaves, respectively. In a young green OD Cry2Aa2 transgenic leaf (Fig. 6A), labeled Cry2Aa2 occupies a significant amount of the chloroplast, but no crystalline structures are observed. In a mature green OD Cry2Aa2 transgenic leaf (Fig. 6B), labeled Cry2Aa2 occupies a larger amount of the chloroplast than the younger leaf, resulting in crystals. These cuboidal crystals are essentially identical to those expressed in wild-type Cry2Aa2 crystals, or recombinantly in *Bt* or *E. coli*[18]. In an old bleached OD Cry2Aa2 transgenic leaf (Fig. 6C), labeled Cry2Aa2 maintains the crystalline structure and occupies the highest volume of the chloroplast observed, despite being bleached and senescent. These findings correlate with OD Cry2Aa2 ELISA results. In young developing leaves, OD Cry2Aa2 begins accumulating (34.9%), folds Cry2Aa2 into a cuboidal configuration in mature leaves occupying more cell volume (45.3%), and maintains this cuboidal structure and volume in old leaves (46.1%). Essentially, as the transgenic OD Cry2Aa2 plant ages, OD Cry2Aa2 is accumulated, folded, and maintained.

Figure 6D is a mature green OD Cry2Aa2 transgenic leaf showing crystal formation with no immunogold label. This probably occurs because as the Cry2Aa2 is folded by the putative chaperonin, epitopes are concealed thereby decreasing labeling. Crystal formation in Figure 6D would cause the OD Cry2Aa2 to pellet after centrifugation as seen in SDS–PAGE (Fig. 3, lane 5). In EM analysis of mature leaves expressing SG Cry2Aa2 (Fig. 6E), protein aggregation is observed, although no crystalline folding is seen. Cry2Aa2 immunogold labeling occurs in an area of much lower density than is seen in OD Cry2Aa2 transgenic plants suggesting lower expression. These results also correlate with ELISA (0.36% in SG Cry2Aa2 in mature leaves). There is no localized antibody observed in untransformed tobacco (Fig. 6F).

Transgenic phenotypes. Phenotypes of OD Cry2Aa2 transgenic plants are not morphologically different from SG Cry2Aa2 transgenic plants (data not included). Therefore, higher levels of expression and accumulation of Cry proteins did not visibly influence their phenotype. Both transgenic plants flowered and set seeds. Characterization of OD Cry2Aa2 T_1 transgenic plants for stable integration and transmission of foreign genes has been shown earlier (Fig. 2).

Discussion

Introducing blocks of foreign genes in a single operon would avoid complications inherent in putting one gene at a time into random locations in the nuclear genome[1]. Cloning several genes into a single T-DNA does not avoid the compounded variable expression problem encountered in nuclear transgenic plants[1]. This study shows that a bacterial operon can be expressed in a single integration event. Expression of multiple genes through a single transformation event opens the possibility of expressing foreign pathways or pharmaceutical proteins involving multiple genes. Also, formation of crystals of

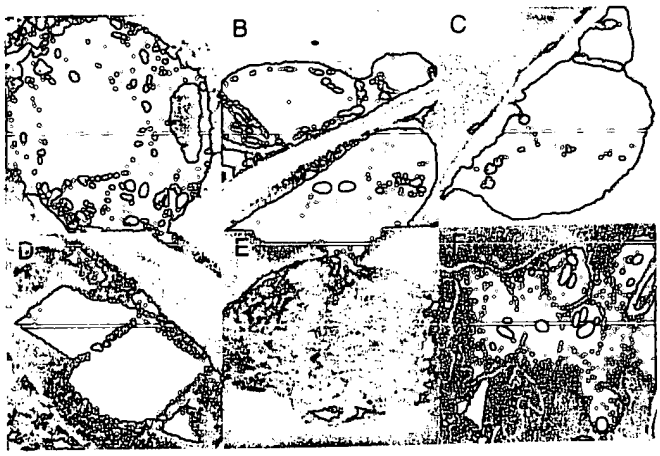

Figure 6. Transmission electron micrographs. Operon-derived Cry2Aa2 leaf sections in young (A), mature (B, D), and old, bleached leaf (C). (E) Single gene-derived Cry2Aa2 mature leaf; (F) mature untransformed leaf.

foreign proteins provides a simple method of purification by centrifugation. Plants transformed with the *cry*2Aa2 operon show a large accumulation and improved persistence of the expressed insecticidal protein(s) throughout the life of the plant. This is most likely because of the folding of the insecticidal protein into cuboidal crystals, thereby protecting it from proteases. The folded crystals may improve the safety of the *Bt* transgenic plants. In contrast to currently marketed transgenic plants that contain soluble Cry proteins, folded protoxin crystals will be processed only by those target insects that have a highly alkaline gut environment. For example, Bradley *et al.*[17] have shown that there is a more than 30-fold concentration difference in the activity of soluble and crystalline Cry protein and that this difference was due to the host midgut alkaline environment. In addition, absence of insecticidal protein in transgenic pollen eliminates toxicity to nontarget insects through pollen. Expression of the *cry*2Aa2 operon in chloroplasts provides a model system for hyperexpression of foreign proteins in a folded configuration, which should enhance their stability and facilitate single-step purification.

Exp rim ntal protocol

Bombardment and selection of transgenic plants. Sterile leaves were bombarded using the Bio-Rad PDS-1000/He biolistic device as described[13,14]. Bombarded leaves were subjected to two rounds of selection on RMOP medium containing 500 µg/ml of spectinomycin to regenerate transformants.

PCR Analysis. DNA was extracted from leaves using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA). PCR was done using the Perkin Elmer Gene Amp PCR System 2400 (Perkin Elmer, Chicago, IL). All PCR reactions were performed using the Qiagen Taq DNA Polymerase Kit and with primers reported earlier[11,12,15]. Samples were run for 30 cycles with the following sequence: 94°C for 1 min, 65°C for 1.5 min, and 72°C for 3 min. PCR products were separated on 0.8% agarose gels.

Southern blot analysis. Plant DNA was digested with *Bgl*II and transferred to a nylon membrane by capillary action. The 0.81 kb probe was generated by digesting pLD-CtV2 vector DNA with *Bam*H1/*Bgl*II and was labeled with ^{32}P using the ProbeQuant G-50 Micro Columns (Amersham, Arlington Heights, IL). The probe was hybridized with the nylon membrane using the Stratagene QUICK-HYB hybridization solution and protocol (Stratagene, La Jolla, CA).

SDS–PAGE Analysis. Leaf material (600 mg) was ground to a powder in liquid nitrogen. Protein extraction buffer from the Cry2Aa2 plate kit from Envirologix (Portland, ME) used for quantification was added to the powder, and further grinding was done. The mixture was centrifuged at 4°C at 13,000 *g* for 20 min. The supernatant was removed, boiled in sample buffer, and loaded on a 10% SDS–PAGE gel. The pellet was resuspended in 50 mM NaOH and centrifuged at 4°C at 5,000 *g* for 5 min to pellet cell debris. The supernatant was removed, boiled in sample buffer, and loaded on a 10% SDS–PAGE gel at 200 V for 4 h. The DC protein assay by Bio-Rad (Hercules, CA) was used to determine total soluble and pellet protein concentration following the manufacturer's protocol.

ELISA. A Cry2Aa2 plate kit from Envirologix was used for this experiment. Approximately 20 mg of leaf were ground in 100 µl of 50 mM NaOH to solubilize Cry proteins. Transgenic leaf extracts were diluted to fit in the linear range of the provided Cry2aA2 standard. The µQuant microtiter plate reader from Bio-Tek (Highland Park, VT) read the plate at 450 nm. A 1 p.p.m. Cry2Aa2 standard was supplied by the kit and was used in the linear range between 200 and 1000 ng for quantification. The DC protein assay by Bio-Rad was used to determine total soluble protein concentration following the manufacturer's protocol.

Insect bioassays and transmission electron microscopy. Leaf disk bioassays were conducted as reported[15]. All insects were reared on typical lepidopteran artificial diet before use[20, 21]. Immunogold-labeled EM was carried out as described[22]. Sections were examined in a Zeiss EM 10 transmission electron microscope at 60 kV.

Acknowledgments

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Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology

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Chloroplast genomes defied the laws of Mendelian inheritance at the dawn of plant genetics, and continue to defy the mainstream approach to biotechnology, leading the field in an environmentally friendly direction. Recent success in engineering the chloroplast genome for resistance to herbicides, insects, disease and drought, and for production of biopharmaceuticals, has opened the door to a new era in biotechnology. The successful engineering of tomato chromoplasts for high-level transgene expression in fruits, coupled to hyper-expression of vaccine antigens, and the use of plant-derived antibiotic-free selectable markers, augur well for oral delivery of edible vaccines and biopharmaceuticals that are currently beyond the reach of those who need them most.

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Chloroplast transformation is an environmentally friendly approach to plant genetic engineering that minimizes out-crossing of transgenes to related weeds or crops [1,2] and reduces the potential toxicity of transgenic pollen to non-target insects [3]. Because the plastid genome is highly polyploid, transformation of chloroplasts permits the introduction of thousands of copies of foreign genes per plant cell, and generates extraordinarily high levels of foreign protein [3]. Chloroplast transformation vectors use two targeting sequences that flank the foreign genes and insert them, through homologous recombination, at a precise, predetermined location in the organelle genome (Fig. 1). This results in uniform transgene expression among transgenic lines and eliminates the 'position effect' often observed in nuclear transgenic plants. Gene silencing, frequently observed in nuclear transgenic plants, has not been observed in genetically engineered chloroplasts. The ability to express foreign proteins at high levels in chloroplasts and chromoplasts, and to engineer foreign genes without the use of antibiotic resistant genes [4,5], make this compartment ideal for the development of edible vaccines [6]. Moreover, the ability of chloroplasts to form disulfide bonds and to fold human proteins has opened the door to high-level production of biopharmaceuticals in plants [7]. Furthermore, foreign proteins observed to be toxic in the cytosol are non-toxic when accumulated within transgenic chloroplasts [6,8]. Chloroplast and nuclear genetic engineering are compared in Table 1.

Brief history of chloroplast genetic engineering

When the concept of chloroplast genetic engineering was developed in the 1980s, it was possible to introduce isolated intact chloroplasts into protoplasts and regenerate transgenic plants. Therefore, early investigations of chloroplast transformation in vascular plants focused on the development of chloroplast systems capable of efficient, prolonged protein synthesis and expression of foreign genes [9]. The development of the gene gun as a transformation device by John Sanford (reviewed in Ref. [10]), enabled plant chloroplasts to be transformed without the use of isolated plastids. Chloroplast genetic engineering was accomplished through autonomously replicating chloroplast vectors in dicot plastids [11] and transient expression in monocot plastids [12], and by stable integration of selectable marker genes into the chloroplast genomes of *Chlamydomonas reinhardtii* and tobacco using the gene gun [13,14]. However, it is only recently that genes conferring agronomically valuable traits have been introduced via chloroplast genetic engineering. For example, integrating the *cry* genes into the chloroplast genome generated plants that were insecticidal to *Bacillus thuringiensis* (*Bt*)-sensitive [15] and highly resistant insects [16]. Chloroplasts have also been engineered recently to generate plants tolerant to bacterial and fungal diseases [17], drought [8] or herbicides [2,18–20]. Transgenes recently engineered via the chloroplast genome are listed in Table 2.

Exceptionally high accumulation of foreign proteins (up to 46% of total soluble protein) has been reported recently for chloroplast transgenes [3]. This feature should make the compartment ideal for low-cost production of biopharmaceuticals. Stable expression of a pharmaceutical protein in tobacco chloroplasts was first reported for GVGVP, a protein-based polymer with varied medical applications [21], and subsequently for human somatotropin [7]. Additionally, chloroplast genomes have been engineered to express pharmaceutical peptides [17], human serum albumin (HSA), monoclonals (H. Daniell *et al.*, www.publish.csiro.au/books/bookpage.cfm?PID=3051&TXT=DES#DES) and antigens composed of oligomeric proteins with stable disulfide bridges [6]. Several recent advances should make chloroplasts even more attractive as

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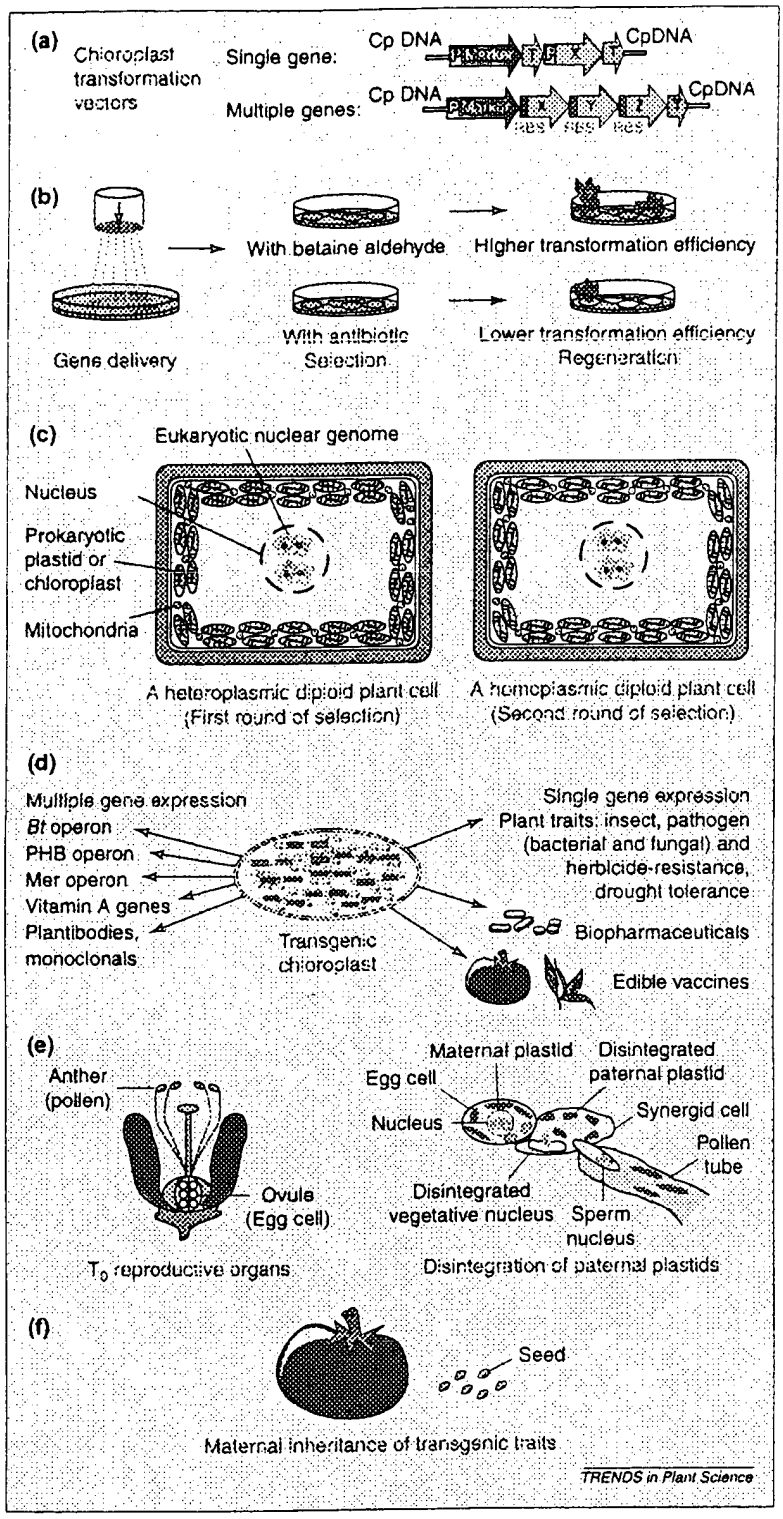

Fig. 1. Various steps in chloroplast genetic engineering. (a) In single-gene (X) chloroplast transformation vectors, coding sequences are flanked by independent 5′ and 3′ regulatory regions, which encompass promoters (P) and terminators (T), respectively. In multiple gene (X, Y, Z) constructs, a single promoter (P) regulates expression of the operon, and individual ribosome binding sites (RBS) are engineered upstream of each open-reading-frame. (b) Gene delivery is usually performed by particle bombardment of chloroplast vectors. Antibiotic selection is performed with spectinomycin or streptomycin, whereas betaine aldehyde is used for plant-derived antibiotic-free selection. (c) The first round of selection generally results in heteroplasmy (left) whereas the second round achieves homoplasmy (right). (d) High-level expression of all the single and multiple gene traits listed here have been achieved, except for PHB and vitamin A operons. This is because of high ploidy of transgenes (5000–10 000 copies per cell). (e) In T_0 reproductive organs, after meiosis, haploid egg and sperm cells are formed (left). The zygote contains only maternal plastids because the paternal plastids disintegrate in the synergid cell (right). Maternal inheritance of transgenes offers biological containment because of lack of gene flow through pollen. Transgenic pollen generally does not contain the foreign gene product. (f) When transgenic seeds are germinated on selectable agents, 100% germination is observed. In contrast with Mendelian segregation of traits, chloroplast transgenes are maternally transmitted without any segregation.

biopharmaceutical reactors. These include engineering multiple foreign genes as operons [3]; transformation without use of antibiotic markers [4,5]; elimination of resistance genes subsequent to transformation [18,22,23]; and the transformation of edible crops such as potato and tomato [24,25]. This review highlights these and other recent achievements.

Engineering the chloroplast genome for herbicide resistance

Glyphosate is a potent, broad-spectrum herbicide that is highly effective against grasses and broad-leaf weeds. Glyphosate works by competitive inhibition of an enzyme in the aromatic amino acid biosynthetic pathway, 5-enol-pyruvyl shikimate-3-phosphate synthase (EPSPS). Unfortunately, like most commonly used herbicides, glyphosate does not distinguish crops from weeds, thereby restricting its use. Engineering crop plants for resistance to the herbicide is a standard strategy to overcome the lack of herbicide selectivity. However, this approach raises the concern that if the engineered resistance gene escapes via pollen dispersal, it might result in resistant weeds or might cause genetic pollution among other crops [26,27]. Engineering foreign genes through chloroplast genomes (which are maternally inherited in most crops) offers a solution to this problem. Although pollen from plants with maternal chloroplast inheritance can contain metabolically active plastids, the plastid DNA is lost during pollen maturation and hence is not transmitted to the next generation [4]. In addition, the target proteins for many herbicides are compartmentalized within the chloroplast. The chloroplast genome can be engineered to confer herbicide resistance by expressing a petunia EPSPS nuclear gene via the chloroplast genome [2]. The resultant transgenic plants are resistant to tenfold higher levels of glyphosate than the lethal dosage, and the transgene is maternally inherited.

Recently, the *Agrobacterium* EPSPS gene (*C4*) was expressed in tobacco plastids and resulted in 250-fold higher levels of the glyphosate-resistant C4 protein than were achieved via nuclear transformation [19]. Even though C4 expression in plastids was enhanced more than nuclear expression levels, field tolerance to glyphosate

Table 1. Comparison of chloroplast and nuclear genetic engineering

Transgenic	Chloroplast genome	Nuclear genome
Transgene copy number	10–100 plastid genome (single circular chromosome) copies per plastid, and 10–100 plastids per cell depending upon age and type of tissue, resulting in many transgene copies (up to 10 000) per cell	Chromosome number is species-specific, but two copies of each of many chromosomes present per cell results in a few copies of the transgene per cell
Level of gene expression	Polyploidy results in abundant transgene transcripts and a high accumulation of foreign proteins (up to 47% of total soluble protein)	Gene regulation determines the rate of transcription, and accumulation of foreign protein is often a limitation
Gene arrangement and transcription	Genes are often arranged in operons and transcribed into polycistronic RNA so that multiple transgenes can be introduced and expressed in a single transformation event	Each transgene is independently inserted into the chromosome and transcribed into a monocistronic mRNA
Position effect	Site-specific insertion through two homologous recombination events eliminates position effects on transgene expression	Random insertions result in variable transgene expression levels
Gene silencing	Not reported	Gene silencing results in decrease or elimination of transgene expression. Both transcriptional and post-transcriptional gene silencing have been reported
Gene containment	Maternal gene inheritance in most crop plants results in natural gene containment	Paternal transgene inheritance results in out-crossing among crops and weeds
Folding and disulfide bond formation	Chloroplasts form disulfide bonds and correctly fold human proteins, making them ideal for development of edible vaccines, pharmaceuticals and plantibodies	For disulfide bond formation, proteins are targeted to the endoplasmic reticulum
Toxicity of foreign proteins	Adverse effects of toxic proteins might be minimized by chloroplast compartmentalization	Toxic proteins accumulating within the cytosol might result in serious pleiotropic effects
Transgenic lines	Uniform gene expression	Highly variable gene expression
Homogeneity at ploidy level	Chloroplast transgenic lines are mostly homoplasmic (all genome copies are homogeneous for the transgene). Homoplasmy is mostly achieved by repetitive selection and regeneration	Nuclear transgenic lines are either heterozygous or homozygous. Homozygosity is achieved either by selfing or crossing

remained the same, showing that higher levels of expression do not always proportionately increase herbicide tolerance. In two different studies, transgenic tobacco plants expressing *bar* genes via the chloroplast genome exhibited field-level tolerance to phosphinothricin (PPT) [18,20]. In this case, even plants with the lowest levels of *bar* expression were resistant to the highest levels of PPT tested, and no pollen transmission of the transgene was detected. However, high-level expression of *bar* genes in the chloroplast was not sufficient to allow direct selection of chloroplast transformants on medium containing PPT [20].

Engineering bacterial operons via chloroplast genomes

Typical plant nuclear mRNAs are monocistronic (i.e. only one polypeptide chain is translated per messenger molecule). This poses a serious drawback when engineering multiple genes [28]. For example, to express the polyhydroxybutyrate polymer or Guy's 13 antibody, single genes were first introduced into individual plants, which were then backcrossed to reconstitute the entire pathway or the complete protein [29,30]. Similarly, in a seven-year effort, three genes were introduced in rice to generate a biosynthetic pathway for β-carotene expression [31]. By contrast, most chloroplast genes are co-transcribed as polycistronic RNAs (i.e. they encode multiple proteins that are separately translated from the same mRNA molecule), which are subsequently processed to form translatable transcripts [28]. Therefore, introduction of multiple chloroplast transgenes arranged in an operon should allow expression of entire pathways in a single transformation event.

Recently, the *Bt cry*2Aa2 operon was used as a model system to test the feasibility of multigene operon expression in engineered chloroplasts [3]. *Cry*2Aa2 is the distal gene of a three-gene operon. The open-reading-frame immediately upstream of *cry*2Aa2 encodes a putative chaperonin that facilitates the folding of Cry proteins into stable cuboidal crystals [32]. Operon-derived Cry2Aa2 protein accumulates in transgenic chloroplasts as cuboidal crystals, to a level of 45.3% of the total soluble protein and remains stable even in senescing leaves (46.1%). These data suggest that chaperonin-mediated folding might assist large-scale production of foreign proteins within chloroplasts. The crystals enhance protein stability and facilitate single-step

Table 2. Foreign gene expression in chloroplasts of higher plants[a]

Genes and use	Gene products and use	Refs
Selectable markers and reporters		
aadA	Aminoglycoside-3′-adenylyltransferase	[14]
nptII	Neomycin phosphotransferase	[52]
codA	Cytosine deaminase	[53]
BADH	Betaine aldehyde dehydrogenase	[4]
uidA	β-glucoronidase	[12]
cat	Chloramphenicol acetyl transferase	[9,11]
gfp	Green fluorescent protein	[24,54]
aadA:gfp	Selectable or screenable fusion protein	[47]
Plant traits: herbicide resistance		
aroA	Glyphosate resistance	[2,19]
bar	Bialaphos resistance	[18,20]
Insect resistance		
Cry1Ac	*Bacillus thuringiensis* (*Bt*) toxin	[15]
Cry2Aa2	*Bacillus thuringiensis* (*Bt*) toxin	[16]
Cry2Aa2 operon	*Bacillus thuringiensis* (*Bt*) toxin	[3]
Pathogen resistance		
msi-99	Bacterial, fungal resistance	[17]
Drought or salt tolerance		
tps1	Trehalose phosphate synthase	[8]
BADH	Betaine aldehyde dehydrogenase	[4]
Amino acid biosynthesis		
EPSPS	5-enol-pyruvyl shikimate-3-phosphate synthase	[2,19]
ASA2	Anthranilate synthase (AS) α-subunit	[55]
Phytoremediation		
mer A	Mercuric ion reductase	[b]
mer B	Organomercurial lyase	[b]
Non-plant traits: biopharmaceuticals		
hST	Human somatotropin	[7]
HSA	Human serum albumin	[c]
msi 99	Anticancer, lytic antibiotic	[17]
proinsulin	Human insulin α, β chains	[d]
IFN α 5	Human interferon α5	[e]
Monoclonals		
Guy's 13	For dental caries against *Streptococcus mutans*	[c]
Biomedical polymer		
gvgvp-120	Bioelastic protein-based polymer	[21]
Edible vaccines		
ctxB	Cholera toxin β-subunit	[6]

[a]Only the first reports are included here, unless a variant or synthetic gene was used in subsequent investigations.
[b]O. Ruiz, MS thesis, University of Central Florida, 2001.
[c]H. Daniell *et al.*, www.publish.csiro.au/books/bookpage.cfm?PID=3051&TXT=DES#DES
[d]O. Carmona-Sanchez, MS thesis, University of Central Florida, 2001.
[e]M. Torres, MS thesis, University of Central Florida, 2001.

purification. This is the highest level of foreign gene expression ever reported in transgenic plants, killing insects that are exceedingly difficult to control. Importantly, this study also showed that there was no insecticidal protein present in pollen, thus eliminating potential harm to non-target insects. This first demonstration of bacterial operon expression in transgenic plants opens the door to engineer novel pathways in a single transformation event. Subsequently, the *mer* operon has been used to express mercuric ion reductase and organomercurial lyase to achieve phytoremediation of mercury (O. Ruiz, MS thesis, University of Central Florida, 2001).

Engineering the chloroplast genome for pathogen resistance

Because plant diseases have plagued global crop production, it is highly desirable to engineer plants that are resistant to pathogenic bacteria and fungi. Amphipathic peptides such as magainin have known antimicrobial properties [33], but their possible uses in agriculture have not been fully explored yet. Recently, a synthetic antimicrobial peptide (MSI-99) was expressed via the chloroplast genome [17]. MSI-99 is an amphipathic α-helical molecule with affinity for the negatively charged phospholipids found in the outer-membrane of bacteria and fungi. Upon contact with these membranes, individual peptides aggregate to form pores, resulting in microbial lysis. Because of the concentration-dependent action of antimicrobial peptides, MSI-99 was expressed via the chloroplast genome to accomplish high-dose release at the point of infection. *In vitro* and *in planta* assays with T_0, T_1 and T_2 plants confirmed that the peptide was expressed at high levels (up to 21.5% total soluble protein) and retained biological activity against *Pseudomonas syringae*, a major plant pathogen. In addition, leaf extracts from transgenic plants inhibited the growth of pre-germinated spores of three fungal species *Aspergillus flavus*, *Fusarium moniliforme* and *Verticillium dahliae* by >95% compared with untransformed controls – these observations were confirmed by *in planta* assays. Importantly, growth and development of the transgenic plants were unaffected by hyper-expression of MSI-99 within chloroplasts. Because the outer membrane is an essential and highly conserved part of all microbial cells, microorganisms are unlikely to develop resistance against these peptides. Therefore, these results give a new option in the battle against phytopathogens.

Pharmaceutical companies are exploring the use of lytic peptides as broad-spectrum topical and systemic antibiotics. Previous studies have reported that analogs of magainin are effective against hematopoietic, melanoma, sarcoma and ovarian teratoma lines. Cystic fibrosis makes patients susceptible to respiratory infections, especially multidrug-resistant *Pseudomonas aeruginosa*. When MSI-99 was tested against *P. aeruginosa* *in vitro*, cell-free extracts of T_1 chloroplast transgenic plants displayed a 96% level of growth inhibition against *P. aeruginosa* (H. Daniell *et al.*, www.publish.csiro.au/books/bookpage.cfm?PID=3051&TXT=DES#DES). These studies show that MSI-99 has the potential to become an alternative to current antibiotic treatments for microbial infections.

Engineering the chloroplast genome for drought tolerance

Water stress caused by drought, salinity or freezing is a major limiting factor in plant growth and

development. Trehalose is a non-reducing disaccharide of glucose whose synthesis is mediated by the trehalose-6-phosphate (T6P) synthase and trehalose-6-phosphate phosphatase complex in *Saccharomyces cerevisiae*. Because it accumulates under stress conditions such as freezing, heat, salt or drought, there is general consensus that trehalose protects against damage imposed by these stresses [34]. Therefore, engineering high levels of trehalose in plants might confer drought tolerance.

Gene containment in transgenic plants is a serious concern when plants are genetically engineered for drought tolerance because of the possibility of creating drought-tolerant weeds and passing on undesired pleiotropic traits to related crops. To prevent these consequences, it is desirable to engineer crop plants for drought tolerance via the chloroplast genome instead of the nuclear genome. Recently, the yeast *trehalose phosphate synthase* (*TPS1*) gene was introduced into the tobacco chloroplast and nuclear genomes to study the resultant phenotypes [8]. Although the chloroplast transgenic and the nuclear transgenic plants expressed significant TPS1 enzyme activity, chloroplast transgenic plants showed up to 25-fold higher accumulation of trehalose than nuclear transgenic plants. Nuclear transgenic plants (T_0) with significant amounts of trehalose accumulation exhibited a stunted phenotype, sterility and other pleiotropic effects, whereas chloroplast transgenic plants (T_1, T_2 and T_3) grew normally and had no visible pleiotropic effects. Chloroplast transgenic plants also showed a high degree of drought tolerance by growing in 6% polyethylene glycol, whereas respective control plants were bleached. Air-dried chloroplast transgenic seedlings and transgenic plants under extreme drought (not watered for 24 days) successfully rehydrated, whereas similarly treated control plants died. Investigations have confirmed that trehalose functions by protecting the integrity of biological membranes rather than regulating water potential. Therefore, this study shows that compartmentalization of trehalose within chloroplasts confers drought tolerance without undesirable phenotypes.

Engineering the chloroplast genome without antibiotic resistance genes

One disadvantage of chloroplast genetic engineering technology is the use of the antibiotic resistance gene, *aadA*, as a selectable marker. The *aadA* gene product inactivates its selective agents, spectinomycin and streptomycin, by transferring the adenyl moiety of ATP to the antibiotics. Because these antibiotics are commonly used to control bacterial infection in humans and animals, there is concern that their over-use might lead to the development of resistant bacteria. Therefore, several studies have explored strategies for engineering chloroplasts that are free of antibiotic-resistance markers. The spinach betaine aldehyde dehydrogenase (BADH) gene has been developed as a plant-derived selectable marker to transform chloroplast genomes [4]. The selection process involves conversion of toxic betaine aldehyde (BA) by the chloroplast-localized BADH enzyme to non-toxic glycine betaine, which also serves as an osmoprotectant [35]. Because the BADH enzyme is present only in chloroplasts of a few plant species adapted to dry and saline environments [35,36], it is suitable as a selectable marker in many crop plants. The transformation study showed that BA selection was 25-fold more efficient than spectinomycin, exhibiting rapid regeneration of transgenic shoots within two weeks. This is the first report of chloroplast engineering without the use of antibiotic resistance genes. Use of selectable markers that are naturally present in spinach, in addition to gene containment, should ease public concerns over genetically modified crops.

Another approach to develop marker-free transgenic plants is to eliminate the antibiotic resistance gene after transformation. Strategies have been developed to remove genes using endogenous chloroplast recombinases that delete the marker genes via engineered direct repeats [18]. Early experiments with *Chlamydomonas reinhardtii* showed that it is possible to exploit these recombination events to eliminate introduced selectable marker genes. Homologous recombination between two direct repeats, engineered to flank a selectable marker, enabled marker removal under non-selective growth conditions [37]. A similar approach applied in tobacco was effective in generating *aadA*-free T_0 transplastomic lines while leaving a third unflanked transgene, *bar*, in the genome to confer herbicide resistance [18]. Recently, another strategy to eliminate selectable marker genes has been developed, using the P1 bacteriophage CRE-lox site-specific recombination system. In two separate studies, a marker gene flanked by lox-sites was introduced into the tobacco chloroplast genome. Its removal was subsequently induced by expressing the CRE protein from the nucleus [22,23]. These reports show that efficient removal of selectable marker(s) from chloroplast genomes is feasible.

Engineering the chloroplast genome to overproduce biopharmaceuticals

Although pharmaceutical proteins have been synthesized from plant nuclear transgenes, expression levels (particularly of human proteins) are generally disappointingly low [38]. Chloroplasts, with their highly polyploid genomes offer an ideal compartment for overproduction of foreign proteins. An additional significant advantage of using chloroplasts is their potential to process eukaryotic proteins, including folding and formation of disulfide

bridges. Such folding and assembly can minimize the need for expensive *in vitro* processing of pharmaceutical proteins after their extraction. For example, 60% of the total production cost of human insulin is associated with *in vitro* processing [39].

Somatotropin (hST), a human therapeutic protein, is used in the treatment of hypopituitary dwarfism, Turner syndrome, chronic renal failure and HIV wasting syndrome. hST has been expressed in tobacco chloroplasts to levels of between 0.2 and 7.0% of total soluble protein in plants, depending upon the plastid translation signals used [7]. Chloroplast-expressed hST was shown to be correctly disulfide-bonded and biologically active. Recently, we have achieved high-level accumulation of HSA in transgenic tobacco chloroplasts in spite of the protein's extreme susceptibility to proteolytic degradation (H. Daniell *et al.*, www.publish.csiro.au/books/bookpage.cfm?PID=3051&TXT=DES#DES). HSA accounts for 60% of the total protein in blood serum and is the most widely used intravenous protein in several human therapies. In transgenic chloroplasts, HSA was expressed between 0.02% and 11.10% total soluble protein depending upon the regulatory signals used. HSA accumulation is so high that inclusion bodies increase the size of the transgenic chloroplasts, facilitating purification by centrifugation. This is the highest level of pharmaceutical protein expressed in transgenic chloroplasts, 500-fold higher than previous reports of HSA expression in nuclear transgenic plants.

Engineering the chloroplast genome to develop edible vaccines

Another major cost of biopharmaceutical production lies in purification; for example, in insulin production, chromatography accounts for 30% of the production cost and 70% of the set-up cost [39]. Therefore, oral delivery of properly folded and fully functional biopharmaceuticals should significantly cut down the production cost. Bioencapsulation of pharmaceutical proteins within plant cells offers protection against digestion in the stomach but allows successful delivery to the target tissues [40].

The β subunits of enterotoxigenic *E. coli* (LTB) and cholera toxin of *Vibrio cholerae* (CTB) are candidate vaccine antigens. Integration of an unmodified CTB gene into the tobacco chloroplast genome results in accumulation of up to 4.1% of total soluble leaf protein as functional CTB oligomers (410-fold higher than the unmodified LTB gene expressed via the nuclear genome) [6]. In addition, binding assays confirm that chloroplast-synthesized CTB binds to the intestinal membrane GM1-ganglioside receptor, indicating correct folding and disulfide bond formation of the plant-derived CTB pentamers. Increased production of an efficient transmucosal carrier molecule and delivery system, such as CTB, in transgenic chloroplasts makes plant-based oral pharmaceuticals commercially feasible. Because the quaternary structure of many proteins is essential for their function, this investigation shows the potential for other foreign multimeric proteins to be properly expressed and assembled in transgenic chloroplasts.

Engineering the chloroplast genome of edible crop plants

To exploit the chloroplast compartment for the production of orally delivered pharmaceuticals, it is important to extend chloroplast transformation to edible crops. For this reason, the recent development of chloroplast transformation for both potato [24] and tomato [25] is particularly exciting. Western analysis of transgenic potato tissues revealed that the chloroplast transgene (green fluorescent protein, GFP) was expressed to high levels in leaves (5.00% total soluble protein), but accumulated much lower levels in the microtubers (0.05% total soluble protein). Tomato plants with transformed plastids yielded more encouraging results; although the transgene (*aadA*) expression level was not quantified, western blot analysis showed that the expression levels in tomato fruits was about half that of leaves [25]. This study predicts the feasibility of expressing high-levels of foreign proteins in the plastids of edible plant organs.

Optimizing foreign gene expression in chloroplasts

Regulatory sequences have been analyzed in chloroplasts by fusion to reporter genes. This approach has identified strong chloroplast promoters as well as UTR sequences mediating RNA stability [41]. Because reporter protein levels do not always correlate with steady-state transcript abundance, it is evident that translation is a crucial control step for optimizing protein production. Several studies have revealed a minimal effect of 3′UTR sequences on chloroplast gene translation [42] although, recently, the petD 3′UTR was shown to influence reporter protein expression [43]. By contrast, many 5′UTRs mediate enhanced translation of downstream open-reading-frames [44]. One of the most effective of these, the tobacco *psbA* 5′UTR, directs strong, light-regulated, translation of chimeric transcripts regardless of which coding sequence, 3′UTR or promoter it is combined with [42].

Additional translational-enhancing elements have been engineered from non-plant sequences. For example, the bacteriophage T7 gene *10* leader sequence (G*10*L), which enhances translation of foreign genes in bacteria, has the same effect in chloroplasts [7,19,21,45]. Accumulation of a herbicide-tolerant EPSPS protein was increased 200-fold when its chloroplast transgene included the G*10*L sequence [19]. This translational enhancement was attributed to complementarity between the G10L ribosome-binding site (rbs) and

the anti-rbs on chloroplast 16S rRNA [7,19]. However, complementarity of a foreign transcript to 16S rRNA is not always beneficial. The first 24 nucleotides of the T7 gene *10* coding sequence are partially complementary to the chloroplast 16S rRNA. In bacteria, genes with this feature are highly translated. By contrast, in chloroplasts, fusion of these 24 nucleotides to a reporter sequence reduced its translation, and mutagenizing these nucleotides to improve their complementarity to the16S rRNA actually reduced reporter gene transcript and protein levels [45]. Therefore, the 5′ end of a transcript's coding region influences expression of chloroplast transgenes. In support of this conclusion, fusion of the 14 N-terminal amino acids of the chloroplast *rbcL* and *atpB* genes to a reporter sequence resulted in different levels of reporter protein that did not reflect differences in transcript abundance [46]. Furthermore, silent mutations in the fused N-terminal coding sequences were found to decrease reporter protein accumulation without influencing RNA level [46]. In a separate study, fusion of sequences encoding the 14 N-terminal amino acids of GFP to a synthetic EPSPS gene increased EPSPS protein accumulation 33-fold without influencing transcript levels [19]. Therefore, when designing foreign genes for maximal expression in chloroplasts, one must optimize not only 5′UTR elements but also sequences downstream of the initiation codon. By changing these sequences it is possible to manipulate levels of foreign protein over an extraordinary 10 000-fold range [19].

Current limitations of chloroplast genetic engineering

One barrier to developing plastid transformation for crop plants, including cereals, has been their regeneration from non-green embryonic cells (containing proplastids) rather than leaf cells (containing chloroplasts). Plastid transformation in rice has been shown using vectors containing a translational fusion of *aadA* with *gfp* [47]. Inspection of streptomycin-resistant rice shoots by confocal microscopy revealed a few proplastids with GFP fluorescence. Although this report only showed heteroplasmy, it represents a step in the right direction towards monocot plastid transformation. A major limitation in accomplishing this goal might be the low level of marker gene expression in non-green plastids because of low genome copy number and low rates of protein synthesis. Identification of promoters and UTRs active in non-green tissues should help to overcome this limitation.

Another limitation is the lack of information on genome sequences for several important crop species to locate intergenic sequences for integration of transgenes. Because of the conservation of the plastid genome sequence across many plant species, the concept of using targeting sequences from one species to transform the plastid genome of another unknown species was developed [2]. Both potato [24] and tomato [26] were transformed using chloroplast vectors with tobacco targeting sequences, and tobacco was transformed with petunia targeting sequences [17].

Yet another limitation is the challenge of delivering foreign DNA through the double plastid membrane. Among the methods used to transform plant chloroplasts, only particle bombardment has proven to be efficient in transforming different plant species. PEG treatment of protoplasts has been used to stably transform tobacco chloroplast genomes [48] and although laborious, it is inexpensive when compared to particle bombardment. Microinjection is effective for foreign gene expression in chloroplasts [49], although application of this technology for stable transformation of chloroplast genomes awaits further investigation. Development of new methods of gene delivery systems into chloroplasts should facilitate further progress in this field.

Epilogue

The accomplishments of chloroplast molecular biologists have spearheaded the plant biotechnology revolution, including the cloning and sequencing of the first plant gene [50], the development of the first useful plant trait (herbicide resistance) [50] and the determination of the first complete sequence of a plant genome [51]. Recent success in engineering the chloroplast genome for resistance to herbicides, insects, disease and drought, and for production of biopharmaceuticals, has opened the door to a new era in biotechnology. These recent developments suggest that commercial products of chloroplast genetic engineering are now on the horizon. In spite of being enslaved by the nucleus, this endosymbiont defiantly marches on, leading the biotechnology field in new directions.

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Multigene engineering: dawn of an exciting new era in biotechnology

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Development of a rice variety enriched in provitamin A, the accumulation of polyhydroxybutyrate polyester in *Arabidopsis* nuclear transgenic plants (with enzymes targeted to chloroplasts in both) and the expression of bacterial operons via the chloroplast genome are recent landmark achievements in multigene engineering. Hyper-expression of transgenes has resulted in the formation of insecticidal protein crystals or inclusion bodies of pharmaceutical proteins in transgenic chloroplasts, achieving the highest level of transgene expression ever reported in transgenic plants. These achievements illustrate the potential of multigene engineering to realize benefits of the post-genomic revolution.

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Abbreviations

BADH	betaine aldehyde dehydrogenase
CTB	cholera toxin β subunit
HSA	human serum albumin
PHB	polyhydroxybutyrate
tsp	total soluble protein
UTR	untranslated region

Introduction

A vast majority of agronomic traits are quantitative and are controlled polygenetically. Genetic engineering is now moving from the initial phase of introducing single gene traits (e.g. resistance to herbicide, disease or insects) to multigenic traits [1], coding for complete metabolic pathways, bacterial operons or biopharmaceuticals that require an assembly of complex multisubunit proteins.

Multigene engineering via the nuclear genome involves several challenges. First, generation of transgenic lines expressing individual genes is necessary, because the nuclear genome does not process polycistrons. Second, such independent transgenic lines that harbor transgenes need to be brought together within a single host by repetitive breeding. Unfortunately, this step is complicated by gene silencing and position effects observed frequently in nuclear transgenic plants. Gene silencing has been observed because of the use of repetitive regulatory sequences, integration of multiple copies of the transgene or even as a result of the efficient transcription of transgenes; it occurs both at the transcriptional and post-transcriptional levels [2]. Position effects are caused by the random integration of transgenes into the nuclear genome. Screening of multiple transgenic lines might require the use of different selectable markers at each step. It is remarkable that, despite these technical hurdles, multiple genes have been skillfully engineered via the nuclear genome for the expression of vitamins [3•,4]. However, these efforts have been highly time-consuming; for example, it took seven years to engineer three genes for the expression of provitamin A, even though the authors were fortunate to introduce two genes at once [5].

Fortunately, there are a few alternative approaches to overcome the aforementioned challenges. In one such effort, a series of three genes encoding a polyprotein containing three enzymes were introduced via the nuclear genome. The polyprotein consisted of tobacco vein mottling virus (TVMV) Nla proteinase, and two other reporter genes — namely, acetate kinase and *Tn*9 chloramphenicol acetyl transferase — separated by the TVMV Nla proteinase recognition sequence. The Nla proteinase facilitated separation of the two enzymes, which were independently functional [6]. Although this approach has been used previously for multigene engineering, this study attempted to simultaneously express foreign proteins in the cytosol and chloroplasts. Such polyproteins should be modified, however, to ensure efficient and predictable processing of individual enzymes within chloroplasts.

Besides technical challenges in nuclear multigene engineering, there are unfortunate negative perceptions and environmental concerns about genetically modified food crops. Lack of gene containment owing to the pollen-mediated out-cross of transgenes from nuclear transgenic plants to related crops or weeds has been a major concern [5,7]. In addition, the possibility of insects developing resistance to insecticidal proteins, due to low levels of transgene expression and toxicity of transgenic pollen to non-target insects, has raised environmental concerns for transgenic plants engineered for pest resistance [5,7].

To address some of these environmental concerns and to facilitate multigene engineering in a single transformation step, the chloroplast genome has been targeted to express several foreign genes [8,9]. Compartmentalization and expression of the transgenes in the maternally inherited chloroplasts should help to allay public concerns about gene containment [10,11]. The ability of plants with transgenic chloroplasts to kill insects that developed very high levels of resistance (up to 40 000-fold) against *Bacillus thuringiensis* insecticidal

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proteins should also dispel the fear of insects developing resistance in the field [12]. Further, the lack of toxicity of transgenic pollen to non-target insects is yet another advantage of plants with transgenic chloroplasts [13••]. The capability of breaking expression level barriers without causing harmful effects to the host plant and the ability to engineer multiple genes in a single transformation event, are probably the greatest advantages of chloroplast genetic engineering. Coordinated expression of multiple genes, preferably driven by a single promoter, is especially important for stoichiometric synthesis and assembly of multisubunit proteins like monoclonal antibodies [14]. Observations of nearly 50% foreign protein in the total soluble protein (tsp) [13••] and 17 000% more transcripts in chloroplast transgenic plants than nuclear transgenic plants [15•] assuages the concerns of gene silencing at the transcriptional or post-transcriptional level. Position effects are not observed in chloroplast genetic engineering because of targeted gene integration; several independent chloroplast transgenic lines express foreign proteins to the same level, except for minor physiological variations [16•]. In some cases, manipulation of a pathway or hyper-expression of a transgene is very demanding on nuclear transgenic plants, resulting in deleterious pleiotropic effects including stunted growth and sterility. However, such pleiotropic effects observed in nuclear transgenic plants were alleviated when the same foreign proteins were compartmentalized within transgenic chloroplasts [15•,16•,17]. Other recent developments in chloroplast genetic engineering have been the advent of a plant-derived selectable marker [18••] and transformation of the chloroplast genome of edible plant species, including potato and tomato [19,20•]. This review discusses recent achievements and forecasts the future role of chloroplast and nuclear transformation in multigene engineering of plants.

Nuclear multigene engineering

A significant recent step in multigene engineering has been the development of a rice variety that accumulates provitamin A [3••]. Vitamin A deficiency results in various diseases like night-blindness or even complete blindness. It is estimated that improved vitamin A nutrition can help to prevent over one to two million deaths each year among children aged one to four years. Employing *Agrobacterium*-mediated transformation, three genes essential for the synthesis of the enzymes of the β-carotene biosynthetic pathway were targeted to plastids in rice endosperm using three different vectors. The β-carotene precursor, geranylgeranyl-diphosphate, synthesized in the rice endosperm plastids was efficiently processed into phytoene, by phytoene synthase, and then further converted into lycopene in a reaction catalyzed by phytoene desturase. Lycopene was eventually converted to β-carotene by lycopene β-cyclase, which humans convert into vitamin A. The transgenic rice plants were fertile with no apparent pleiotropic effects.

Another example of nuclear multigene engineering is the expression of three enzymes of the polyhydroxybutyrate (PHB) pathway [21•]. A quadruple construct [22], comprising a selectable marker and three cassettes (each containing one of the three *phb* genes with a plastid targeting signal) flanked by a 35S promoter and *nos* (nopaline synthase) terminator, was used to introduce three genes involved in this pathway. This approach resulted in a large accumulation of PHB (4% fresh weight) fourfold higher than previous reports [17]; however, this had a severe effect on the phenotype of transgenic plants (proportional to PHB accumulation). Lack of gene silencing, in spite of repetitive use of the same regulatory sequences, goes against current understanding of transgene silencing. Unfortunately, the production of PHB polyesters in transgenic plants has not been commercially feasible so far, because of severe effects on growth/fertility and an inability to achieve high expression in large biomass crops.

Chloroplast multigene engineering

The concept of chloroplast transformation, conceived in the mid-80s [23,24], has recently blossomed into a safe and environmentally friendly technology [8,9,25]. When the first transgenes were introduced via the chloroplast genome, it was believed that foreign genes could be inserted only into transcriptionally silent spacer regions, amidst divergent chloroplast genes [26]. However, Daniell *et al.* [10] advanced the concept of inserting transgenes into functional operons and transcriptionally active spacer regions. This approach facilitated the insertion of multiple genes under the control of a single promoter, enabling the coordinated expression of transgenes [13••,15•,16•,27•]. Earlier reports, based on *in vitro* studies of chloroplast mutants, established a definite requirement for the processing of dicistrons to monocistrons before translation [28–30]. To test this hypothesis, multiple transgenes were inserted into the rRNA operon of chloroplast genomes to study their transcription, RNA processing and translation. Contrary to previous reports, the following examples unequivocally demonstrate that polycistrons are efficiently translated in transgenic chloroplasts without any requirement for RNA processing. The fact that several foreign proteins are synthesized in large quantities without any detectable monocistrons support this conclusion.

Expression of a protein-based biomedical polymer as a dicistron in transgenic chloroplasts demonstrated, for the first time, the potential of this technology to engineer biopharmaceuticals [31••,32••]. Recently, human serum albumin (HSA), expressed under the regulation of the optimal chloroplast ribosome-binding site (GGAGG), could not be easily detected (<0.02% tsp) in transgenic chloroplasts. In the past, the same regulatory sequence has resulted in accumulation of large quantities of several other foreign proteins (up to 21% tsp) [27•]. HSA was, however, successfully hyper-

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expressed in transgenic chloroplasts as a dicistron or polycistron, by manipulating the 5′ and 3′ regulatory sequences of the transgene (A Fernandez-San Millan, A Mingo-Castel, H Daniell, unpublished results) [33]. HSA accumulated in such large amounts that inclusion bodies formed and increased the size of transgenic chloroplasts (Figure 1a). HSA inclusion bodies were readily purified by simple centrifugation and solubilized to functional monomers. Regulatory sequences used in this study should serve as a model system for enhancing the expression of foreign proteins that are highly susceptible to proteolytic degradation and in addition should provide major advantages in purification. This study reports the highest level of pharmaceutical protein ever observed in transgenic plants. This is the first report to provide direct evidence for translation of transgene polycistrons, without any requirement for processing to monocistrons. Also, this study identifies a heterologous UTR that could be used in non-green plastids, free of nuclear control. Search for such non-green UTRs have been elusive so far.

To combat a disease like cholera that often assumes epidemic proportions and poses a threat as an agent of bioterrorism, there is a need for producing vaccines on an agricultural scale. Therefore, cholera toxin β subunit (CTB) was expressed in transgenic chloroplasts as a dicistron. As the quaternary structure and disulfide bonds of many pharmaceutical proteins are essential for their function, we demonstrated, using CTB, the assembly of functional oligomers in transgenic chloroplasts. Expression of the native β subunit gene (*ctx* B) was 410-fold higher than in nuclear transgenic plants and there was no pleiotropic effects, in contrast to nuclear transgenic plants that showed stunted growth [16*,33]. Western blot analysis and enzyme-linked immunosorbant assay (ELISA) showed that several independent transgenic lines expressed the same amount of CTB, except for physiological variations [16*]. Engineering CTB in transgenic chloroplasts, along with recent success in the chloroplast transformation of edible crops, and the availability of plant-derived selectable markers, augur well for producing edible vaccines in transgenic chloroplasts on a cost-effective basis [16*,18**,19,20*].

Chloroplast transformation has also been employed to confer resistance to biotic and abiotic stresses. Expression of an antimicrobial peptide, MSI-99, as a dicistron in transgenic chloroplasts was shown to inhibit the growth of several plant pathogens, including *Pseudomonas syringae*, *Aspergillus flavus*, *Fusarium moniliformae*, *Verticillium dahliae* and the multidrug-resistant human pathogen *Pseudomonas aeruginosa*, when tested using *in planta* and *in vitro* assays [27*,34]. Lysis of transgenic chloroplasts at the site of infection resulted in high-dose release of the antimicrobial peptide (800 μg MSI 99, inhibitory concentration 1 μg MSI 99 for 1000 bacterial cells or fungal spores). In another recent report, the integration of a yeast trehalose-6 phosphate synthase (TPS) gene as a dicistron in transgenic chloroplasts was shown to confer drought tolerance, as evidenced by growth of transgenic plants on 6% polyethylene glycol and ability to rehydrate after dehydration [15*,35]. Whereas nuclear transgenic plants accumulating trehalose in the cytosol showed stunted growth, sterility and other pleiotropic effects, chloroplast transgenic plants showed normal growth and physiology and no pleiotropic effects (Figure 2). Chloroplast transgenic plants showed 16 699% more *tps*1 transcripts than the best nuclear transgenic plants, alleviating the possibility of gene silencing in transgenic chloroplasts (Figure 3).

Perhaps the most significant accomplishment, which has made chloroplast transformation technology safe, is the use of a plant-derived selectable marker, betaine aldehyde dehydrogenase (BADH), to obtain chloroplast transgenic plants by expression of a dicistron [18**,36]. The selection process involves conversion of toxic betaine aldehyde to glycine betaine by BADH; glycine betaine also serves as an osmoprotectant. The BADH gene derived from spinach not only eliminates the need for the use of antibiotic resistance genes but is also 25-fold more efficient than antibiotic resistance genes, exhibiting rapid regeneration of transgenic shoots within two weeks. These developments should help to allay public concerns and make genetically modified foods more acceptable.

Ever since chloroplast technology was conceived, it was anticipated that the prokaryotic nature of the organelle should allow the expression of bacterial operons. This promise was realized when expression of the *B. thuringiensis cry*2Aa2 operon in transgenic chloroplasts led to the formation of insecticidal crystals (Figure 1b,c) [13**,37]. The 4.0 kb operon consists of three genes, with *cry*2Aa2 being the distal gene. The open reading frame, *orf*2, immediately upstream of the gene codes for a putative chaperonin that is necessary for folding the protein into cuboidal crystals (that are resistant to proteolytic degradation). Expression of the operon in transgenic chloroplasts resulted in the accumulation of Cry2Aa2 protein at 46.1% of tsp, even in senescing bleached old leaves. Such high levels of insecticidal protein were instrumental in combating insects that are normally difficult to control, including the 10-day old cotton bollworm and beet armyworm. Observed hyper-expression of Cry2Aa2 protein argues against any possibility of gene silencing in transgenic chloroplasts.

The possibility of expressing a pharmaceutical protein, which involves multiple genes, has been explored using the Guy's 13 monoclonal antibody. This antibody against the surface protein of *Streptococcus mutans*, which is the causative agent of dental caries, was successfully expressed and properly assembled in transgenic chloroplasts [14,38]. This is the first demonstration of expression of a multisubunit foreign protein that is assembled with disulfide bridges. Application of Guy's 13 monoclonal antibody to the dental surface prevented

recolonization of the bacterium for up to two years [39]. This multisubunit antibody has been expressed via the nuclear genome by generating independent transgenic lines, followed by subsequent breeding [40]. For commercial application, however, expression levels should be increased further in nuclear transgenic plants.

Phytoremediation is evolving as a safe technology to address the increasing problem of the pollution of soil and water bodies. One of the most toxic pollutants that threatens our health and ecosystem is mercury. In the environment, mercury is rapidly methylated by bacteria producing a 10-fold more toxic organomercurial, owing to its ability to cross lipid membranes [41]. Over 90% of methylmercury is absorbed in blood compared with only 2% of inorganic mercury, causing neurological degeneration in birds, mammals and humans. In photosynthetic organisms, mercury inhibits the oxygen-evolving enzyme (OEE) complex, binds to thylakoid membranes [42] and removes EP33 (one of the proteins of the OEE complex [43]). Mercury reduces the variable fluorescence (which provide a measure of photosynthetic efficiency owing to additional inhibitory sites on the donor side of photosystem II, causing damage to the light-harvesting complexes and structural changes in the antenna pigments that affect the primary photochemistry; mercury also inhibits plastocyanin [44]. Nuclear codon optimized *mer*A (mercury ion reductase) and *mer*B (organomercurial lyase) genes were used to obtain transgenic plants that are resistant to mercury and organomercurials, respectively (up to 10 μM) [45•]. The low level of tolerance observed might result from the low levels of nuclear expression, compounded by the fact that these enzymes were not targeted to chloroplasts, where mercury is most toxic, requiring continuous detoxification. Therefore, the *mer* operon has been expressed via the chloroplast genome to overcome these problems [46•,37].

Conclusions

Plant biotechnology is at the threshold of an exciting new era in which the emphasis is on the introduction of traits that require the manipulation of metabolic pathways or coordinated expression of multisubunit proteins. The development of rice varieties enriched in provitamin A is an early success story in this new era. The chloroplast transgenic approach has facilitated expression of bacterial operons and biopharmaceuticals at unprecedented levels, never before reported in the literature. Accumulation of about 50% of foreign proteins in the total soluble protein in chloroplast transgenic plants resulted in the formation of insecticidal protein crystals or inclusion bodies of biopharmaceuticals. Foreign transcripts in transgenic chloroplasts accumulated 17 000% more than the best nuclear transgenic plants. These exciting achievements not only relieve concerns about gene silencing and position effects, but also eliminate the need for time-consuming breeding to bring multiple transgenes within a single host. In addition, these advances offer several environmentally friendly features including gene containment. The new era will rely heavily on both nuclear and chloroplast multigene engineering technologies to utilize the new knowledge acquired in the post-genomic era for biotechnological applications and to understand complex metabolic pathways.

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This landmark study demonstrates the ability of transgenic chloroplasts to express bacterial operons, opening the door for multigene engineering via the chloroplast genome. Engineering four genes required only a few months to generate transgenic plants. Unprecedented levels of foreign gene expression and protein accumulation (up to 46% tsp) were observed, the highest levels ever reported in transgenic plants. Extremely difficult to

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control insects were killed by these transgenic plants. Such high levels of expression offer direct evidence for the lack of gene silencing in transgenic chloroplasts. The use of a chaperonin protein to fold insecticidal protein into cuboidal crystals protected the foreign protein from proteolytic degradation. In spite of such high levels of accumulation of Cry protein in leaf chloroplasts, transgenic pollen was non-toxic to monarch butterfly larvae. These observations dispel fears of insects developing resistance or negative impact on non-target insects.

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This is the first report of expression of an edible vaccine in transgenic chloroplasts. In addition to hyper-expression of native CTB (410-fold higher than nuclear transgenic plants), this article demonstrates the first assembly of functional oligomers with disulfide bonds in transgenic chloroplasts. Whereas accumulation of CTB was toxic in the cytosol in nuclear transgenic plants, large accumulations in transgenic chloroplasts were non-toxic to cells. Most importantly, this study shows that foreign gene expression is uniform among independent chloroplast transgenic lines, in contrast to nuclear transgenic lines.

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This landmark study demonstrates the ability to engineer the chloroplast genome without the use of antibiotic selection, a serious concern because of the thousands of antibiotic resistance genes present in each cell of transgenic plants. Use of a plant-derived selectable marker, which is more efficient than antibiotic selection, should help allay such public concerns. Most importantly, this report dispels the notion that chloroplast genetic engineering requires the use of non-lethal selectable markers.

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This landmark study demonstrates the expression of the first pharmaceutical protein in transgenic chloroplasts, opening the door for a new area of biotechnological applications. Most importantly, this work developed the concept of universal vectors (use of chloroplast DNA from one genus to transform another genus or family). This concept was subsequently used to transform potato and tomato chloroplast genomes using tobacco vectors. Furthermore, the flanking sequence contained a chloroplast origin of replication, resulting in homoplasmy even in the first round of selection (an important requirement to transform

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monocots). Transgenes were inserted for the first time into transcriptionally active spacer regions (within functional operons) of chloroplast genomes, opening the door for multigene engineering in transgenic chloroplasts using a single promoter. Also, a heterologous untranslated region (UTR; T7 gene 10 from pET11d) was used for the first time in transgenic chloroplasts to enhance translation.

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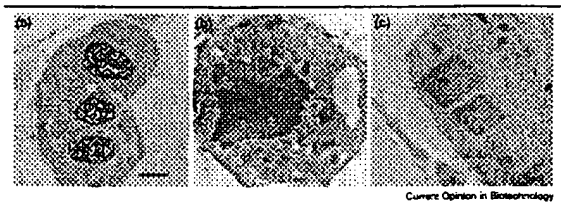

Figure 1
Examples of the highest level of transgene expression. Electron micrographs of the hyper-expression of foreign proteins in transgenic chloroplasts. **(a)** Inclusion bodies of HSA, the most widely used intravenous protein in human therapies. **(b,c)** Immunogold-labeled inclusion bodies and cuboidal crystals of the insecticidal *B. thuringienesis* Cry2Aa2 protein.

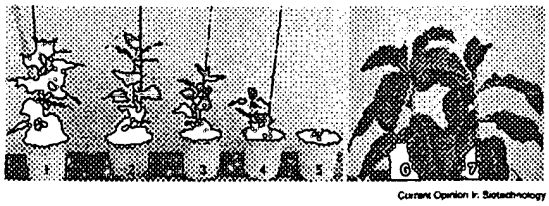

Figure 2
Alleviation of pleiotropic effects. Comparison of the phenotypic effects of trehalose accumulation in the cytosol and chloroplasts of transgenic plants. **(a)** Untransformed wild type (1); nuclear transgenic plants from different, independent transgenic lines (2–5). **(b)** A chloroplast transgenic plant (6); untransformed wild type (7).

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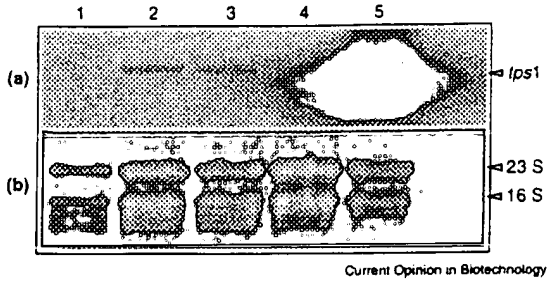

Figure 3

Elimination of gene silencing. Northern blot analysis of nuclear and chloroplast transgenic plants expressing the trehalose phosphate synthase (*tps1*) gene. **(a)** Steady-state transcript levels of *tps1*: (1) untransformed wild type; (2) and (3) highly expressing nuclear transgenic plants; (4) untransformed wild type; (5) chloroplast transgenic plant. **(b)** Ethidium bromide stained total plant RNA to verify equal loading of RNA.